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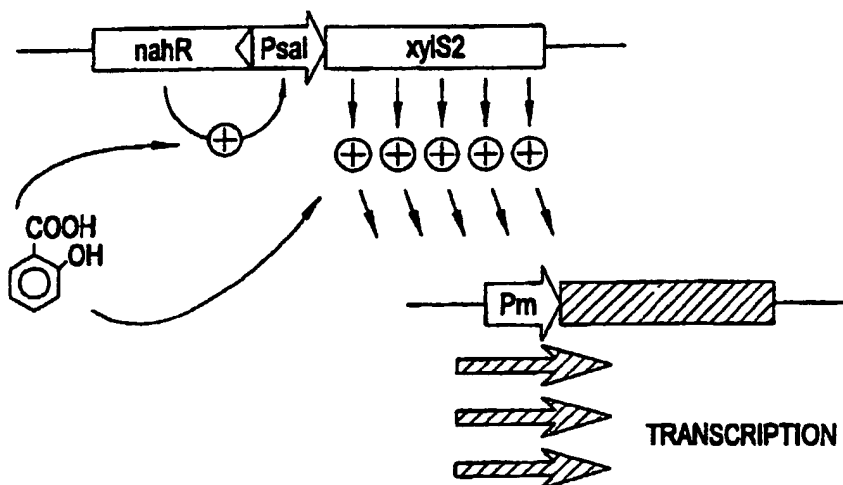
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(54) Title: REGULATED EXPRESSION OF CLONED GENES USING A CASCADE GENETIC CIRCUIT



(57) Abstract: The present invention relates to the design, preparation and use of cascade regulatory circuits for amplification of gene expression. The genetic circuit is based on a plurality (e.g., two or more) of regulatory genes organized in a hierarchical order of expression in a genetic construct or constructs, which can be established in a cell, e.g., a gram-negative bacteria, by means of autoreplicative vectors or by chromosomal insertion. In one embodiment, the genetic construct(s) can be stably maintained in the chromosome without selective pressure, and gene expression induced three orders of magnitude therefrom using economical

**REGULATED EXPRESSION OF CLONED GENES USING A CASCADE**  
**GENETIC CIRCUIT**

**Related Applications**

5           This application claims priority to Spanish Patent Application ES-9901383, filed June 22, 1999 in the name of Cebolla et al., entitled "Tightly regulated overexpression of cloned genes using a cascade genetic circuit", and to Spanish Application ES-200001389, filed May 31, 2000 in the name of Cebolla et al., entitled "Regulated expression of cloned genes using a cascade genetic circuit", each of which applications are herein incorporated  
10 by reference in their entireties including all drawings.

**Field of the Invention**

          The present invention relates to the design of transcriptional cascade circuits to amplify gene expression. It also relates to the use of these systems for the overproduction  
15 of polypeptides such as therapeutic proteins, enzymes, hormones, growth factors, and apolipoproteins in vitro, and in cells, i.e., cell cultures. This has great industrial utility, e.g., in the biotechnology and pharmaceutical industries.

**Background of the Invention**

20           Overexpression of cloned genes is very convenient for production of either recombinant polypeptides or specific cell metabolites for basic research, and the pharmaceutical and biotechnology industries generally. Production of large amounts of cloned genes has traditionally been achieved by combining gene amplification with strong promoters regulated by repressors. However, these conventional strategies typically have  
25 the disadvantages that: 1) maintenance of plasmid expression vectors typically requires selection with antibiotics, giving rise to metabolic burden and additional costs for large scale industrial production (Nilson and Skogman 1986); 2) low level expression is indicated when dealing with toxic proteins, and in avoiding accumulation of mutations in the recombinant protein products themselves (Mertens et al., 1995; Vilette et al.  
30 1995), which is difficult to achieve when the cloned gene is in multicopy due to the multiple copy number of traditional plasmid vectors and the high basal ("leaky") level of

expression of most traditionally used promoters, e.g., *tac* or *trc*; 3) traditional inducers, e.g., IPTG in lac expression systems, are expensive and have a certain degree of toxicity (Figge et al. 1988); 4) high expression of recombinant proteins has been shown to reduce host cell growth rate and, concomitantly, overall protein synthesis (Bentley et al., 1990; 5 Dong et al. 1995), presumably due to increased metabolic burden on the host; and 5) a considerable number of existing expression systems only replicate in *E. coli*, which may limit expression of certain proteins, e.g., those desired to be secreted.

An alternative expression system that fulfills some of the above requirements uses miniTn5 transposon vectors (de Lorenzo and Timmis 1994) to insert heterologous genes 10 into the bacterial chromosome, thereby allowing high stability of expression (Cebolla et al. 1993; Suarez et al. 1997). Suarez in particular describes the stable production of pertussis toxin in *Bordetella bronchiseptica* by miniTn5-mediated chromosomal insertion and expression using a salicylate regulatory system. Salicylate is a benzoate inducer 1000-fold less expensive than IPTG (SIGMA catalog 1998). The system is based on the 15 *nahR* regulatory gene, which encodes a positive regulator activated by salicylate and its target promoter *Psal* (de Lorenzo et al. 1993). However, expression levels obtained are relatively poor (0.1% of total proteins). This low level is likely because the genes are in monocopy in the chromosome.

If yield could be improved while maintaining the advantages of low basal levels, 20 stability, broad host range, and low cost, the *nahR/Psal* regulatory system would have great industrial utility.

### **Summary of the Invention**

We have designed a cascade system that allows 10 to 20 fold greater expression 25 over the standard *nahR/Psal* system while substantially retaining one or more of that system's innate advantages. To achieve this, another regulatory element, *xyIS2* and its target promoter *Pm*, is coupled to *Psal* expression in a cascade circuit. The *xyIS2* regulatory gene responds to the common inducer and has more gene expression capacity than standard *nahR/Psal*. Synergistic activation of the *Pm* promoter by the *XyIS2* 30 transcriptional activator can be achieved by simultaneously increasing the intracellular

concentration and specific activity of activator/regulator in the presence of a common benzoate derivative inducer, e.g., salicylate.

Accordingly, in a first aspect the invention features a cascade genetic circuit comprising one or more nucleic acid constructs encoding a plurality of transcriptional regulators, said encoded regulators arranged in a hierarchical order such that expression of  
5 an upstream regulator from said plurality stimulates expression of a downstream regulator from said plurality; and a final target promoter, said final target promoter responsive in a dose-dependent fashion to a terminal downstream regulator of said plurality of regulators.

In certain preferred embodiments, it may be useful to introduce the final target  
10 promoter alone, e.g., via PCR, into a host genome at designated position to determine effect on expression of the downstream sequence. To do this, it may first be desirable to disable or knock out the native promoter, gene, or nucleic acid sequence. In other preferred embodiments, as described below, heterologous genes and sequences are preferred for use with the cascade circuit and, accordingly, may be introduced.

15 In one especially preferred embodiment, the cascade genetic circuit further comprises a multiple cloning site downstream of the final target promoter.

In another preferred embodiment, the cascade genetic circuit, or at least a portion thereof, is present as a chromosomal integration in a host cell. In a different, not necessarily mutually exclusive embodiment, at least one of said one or more nucleic acid  
20 constructs is present as an autoreplicative plasmid.

In a further embodiment, the cascade genetic circuit, or at least a portion thereof, is responsive to an inducer, preferably an inducer that is capable of inducing the expression of more than one regulator in the cascade. In preferred embodiments, the inducer is a benzoate derivative, preferably, although not necessarily, salicylate.

25 In another aspect, the invention features a cell, tissue, or organism comprising the cascade genetic circuit of any of the preceding claims. Preferably, the cell is selected from the group consisting of procaryotic and eukaryotic cells. As concerns eukaryotic cells, mammalian, insect, yeast, and plant cells are preferred. As concerns procaryotic cells, gram-negative bacterial cells are preferred.

30 In yet another aspect, the invention features methods of regulating the expression of a nucleic acid sequence, comprising establishing a cascade genetic circuit according to any

of the cascade genetic circuit embodiments described above; placing said nucleic acid sequence under control of said final target promoter; and inducing said cascade genetic circuit to stimulate expression of said nucleic acid sequence.

Preferably the nucleic acid sequence encodes a polypeptide selected from the group consisting of enzymes, hormones, growth factors, apolipoproteins, therapeutic proteins, diagnostic proteins, and portions or derivatives thereof. In other preferred embodiments, the nucleic acid sequence encodes an anti-sense molecule, ribozyme, rRNA, tRNA, snRNA, or simply a diagnostic RNA molecule. In certain preferred embodiments, the nucleic acid encodes a reporter gene product useful in diagnostics.

#### Detailed Description of the Invention

By using regulatory genes of the control circuits for the expression of catabolic operons, a cascade expression system for amplifying gene expression was constructed. The system is based on the activation characteristics of the *P<sub>m</sub>* promoter by the XylS2 mutant transcriptional activator. Strength of *P<sub>m</sub>* activation depends on both the amount of XylS2 protein and its specific activity, which is enhanced by the presence of salicylate and other benzoate derivatives. To couple the increase of XylS2 intrinsic activity and XylS2 intracellular concentration, the expression of *xylS2* is under the control of the *P<sub>sal</sub>* promoter and the NahR transcriptional activator, that is also activated in response to common inducers. The synergistic action of both transcriptional regulators lead to 10 to 20-fold amplification of the gene expression capacity with regard to each individual expression system.

One embodiment of the system comprises a cassette having the regulatory genes *nahR/P<sub>sal</sub>::xylS2* flanked by transposable sequences which facilitate stable insertion into the chromosome of a cell, e.g., a gram negative bacteria. A complementary expression module containing the target promoter *P<sub>m</sub>* upstream of a multicloning site for facilitating the cloning of a recombinant DNA is used as part of the system. The expression module can be introduced in multicopy plasmid form or else transferred to the chromosome, e.g., via minitransposon delivery vectors (if either stability of the expression and/or the lowest basal level are desired). To achieve this, the expression module with the *P<sub>m</sub>* promoter and

the heterologous gene/s are preferably flanked by rare restriction sites, e.g., *NotI*, for further cloning utility.

An ideal expression system should be tightly regulated (i.e. to have very low basal level of expression and a high level of expression in the presence of inducer). For large scale fermentations, it is very convenient that the inducer be cheap and that the overexpression of the gene be stable, preferably without selective pressure. The capacity of the culture to reach high biomass should be affected as little as possible, and convenient for use in a broad range of organisms. Salicylate-induced expression using *nahR/Psal* in the chromosome of gram negative bacteria has proven to be very stable and tightly regulated (Suarez et al. 1996). However, the expression level obtained is very low due to single copy presence and limited gene expression capacity.

In contrast, the *xylS/Pm* expression system has shown to have an outstanding range of activity that depends both on the specific activity of the XylS transcriptional regulator and on the intercellular concentration of XylS; manipulation of either affects expression (Kessler et al. 1994).

The transcriptional activity of *Pm* in vivo appears to be non-saturable because, despite overexpression of XylS, expression from *Pm* continues in response to 3-methylbenzoate inducer. However, the expression systems based on the *xylS/Pm* system maintain constant amounts of *xylS* expression, which waste the potential increment in gene expression capacity if *xylS* expression could be made inducible. As the Applicants demonstrate herein, this can be achieved by coupling the expression of *xylS* to another expression system (first system). If the signal that induces transcriptional regulator expression were the same that the one that activates it, the signal could produce synergy in the activation of gene expression from the *Pm* promoter.

The present invention describes this: synergistic signal amplification using coupled expression systems. To use regulators responding to a common signal, *nahR/Psal* was used as a first regulatory system and *xylS2*, a mutant of *xylS* able to respond to salicylate (Ramos et al. 1986), as a second regulatory system. A 1.2 Kb fragment with the *xylS2* gene was cloned by digestion with *HindIII* and partial digestion with *NcoI*, and insertion in the same sites of pFH2 (Table 1). The resultant plasmid pNS2 was digested with *NotI* and the fragment with *xylS2* was inserted into the plasmid pCNB4 (de Lorenzo et al.

1993). This regulator is left under the control of the *nahR/Psal* system and flanked by the insertion sequences of miniTn5. The resultant plasmid pCNB4-S2 (Figure 2a) has a R6K replication origin (Kahn et al. 1979), that can only replicate in strains expressing  $\pi$  protein. *E. coli*  $\lambda$ pir lysogen can express that protein and thus, replicate the miniTn5 delivery  
 5 plasmid (Herrero et al. 1990).

By using the donor strain of miniTn5 vectors S17-1( $\lambda$ pir) one can transfer the regulatory cassette to other gram negative strains using standard biparental conjugation, and select for recipient bacteria using selective markers determined from the minitransposon (Herrero et al. 1990; de Lorenzo and Timmis 1994). To verify that it is an  
 10 insertion by transposition and that the plasmid R6K has not been inserted, the transconjugant colonies are checked for the loss of  $\beta$ -lactamase of the suicide plasmid. Every strain with the regulatory cassette *nahR/Psal::xylS2* would produce the regulatory protein XylS2 in response to salicylate (Figure 1).

This strain can then be used for insertion by conjugation or transformation of a  
 15 second regulatory cassette that contains the *Pm* promoter fused to the heterologous gene of interest. One such construct, pTSPm, is a miniTn5 vector that contains within the insertion sequences a streptomycin resistance gene, the *Pm* promoter, and a *NotI* restriction site (Figure 2a) to insert the heterologous gene.

To construct pTSPm, a fragment with the omega interposon (Fellay et al. 1987)  
 20 was inserted in the *Bam*HI site of the vector pUC18Sfi-Km<sup>R</sup>-*xylS*-*Pm*-Sfi (de Lorenzo et al. 1993) which removed a fragment with Km<sup>R</sup> and *xylS*. The resultant plasmid was digested with *Sfi*I and the biggest fragment was cloned into the pUT backbone of a miniTn5 vector (Herrero et al. 1990) to obtain the plasmid pTSPm. Auxiliary vectors of Table 1 can be used to clone the heterologous genes and then subclone into the *NotI* site of  
 25 pTSPm.

A second expression vector with a ColE1 replication origin (pCCD5) contains the *Pm* promoter upstream of a multicloning site, and a good translation initiation sequence for convenience in cloning heterologous genes (Figure 2b). Plasmid pCCD5 was constructed cloning an *ApoI* fragment containing the *rrnBT1* transcriptional terminator  
 30 produced as a PCR fragment from pKK232-8 with the oligos 5'-GCAAATTTCAGGCATCAAATAA and 5'-GGGAATTCCCTGGCAGTTTATGG,

into the *EcoRI* site of pFH2 (Table 1). Following ligation, a unique *EcoRI* site results and those plasmids with intact MCSs can be selected. The *Pm* promoter was obtained by PCR, using the oligos A-*Pm* (5'-GTGTCAAAATTTGATAGGGATAAGTCC-3') and Pm-E (5'-GCCTGAATTCAGGCATTGACGAAGGCA-3') as primers, and pUC18Sfi-*Km*<sup>R</sup>-*xylS*-*Pm*-Sfi as template. Digestion with *ApoI* (underlined) of the amplified fragment (0.4 Kb) gave a fragment compatible with *EcoRI* termini, but only the extreme of the fragment downstream of the *Pm* promoter regenerated the *EcoRI* site in the junction. Therefore, introduction of the *Pm ApoI* fragment in the proper sense into the *EcoRI* linearized intermediate plasmids, rendered vectors that contained the *Pm* promoter preceeding an intact MCS, with all the key elements flanked by *NotI* sites.

This last feature may allow to clone in mini-Tn5 delivery vectors if monocopy or stability of the expression system are required. There exists up to 8 different makers in miniTn5 vectors with the *NotI* site where the *Pm* fusion may be cloned (de Lorenzo 1994). The resultant vectors may then be inserted into the chromosome of the receptor bacteria by conjugation. Alternatively, cloned genes in pCCD5 under *Pm* control may be transformed in strains with the regulatory cassette in the chromosome to overexpress the heterologous gene from the plasmid.

### **Brief Description of the Drawings**

Figure 1. Schematic representation of a cascade regulatory circuit embodiment. An expression cassette containing the *nahR* gene and the *Psal* promoter controls the expression of *xylS2*. In the presence of a common inducer, NahR activates the expression of *xylS2*. Simultaneous high intracellular levels of XylS2 and stimulation of XylS2 specific transcriptional activity achieves amplified expression levels of a given gene under control of the *Pm* promoter.

Figure 2. Vector scheme embodiments for expression of a genetic circuit embodiment. (a) Constructed miniTn5 vectors. The scheme in the upper part represents the backbone of the suicide plasmid pUT with the replication origin R6K (*oriR6K*), the transposase gene (*tnp\**), the  $\beta$ -lactamase gene that confers ampicillin resistance (*bla*), the mobilization origin (*mob*), and the insertion sequences (I end and O end) represented as horizontal hatched lines. The regulatory cassette in pCNB4-S2 and the expression cassette



in pTSPm are down the pUT scheme. The restriction site *NotI* is rare because it recognizes 8 nucleotides. (b) Scheme of the expression vector pCCD5 (linearized) that contains the *Pm* promoter upstream of a MCS where the heterologous gene can be cloned. It also contains a M13 replication origin that allows to form single stranded DNA plasmids for site directed mutagenesis. As an example, the cloning strategy of the different fragments that form the *lpp*'-'*ompA*'-'*phoA* fusion is shown.

Figure 3. Embodiment scheme of an example of the construction of miniTn5 vectors for an insertion in monocopy of the heterologous genes by transposition. *tnp\** is the transposase gene. The insertion sequences are named I end and O end which are substrate of the transposase. DNA flanked by the insertion sequences can be transposed to the receptor strain. The *NotI* site serves to introduce within the insertion sequences any gene that has been previously cloned in the auxiliary vectors that contain multiple cloning sites flanked by *NotI* sites (Table 1 and for instance, pUC18Not in the scheme)

Figure 4. Comparison of the production of  $\beta$ -galactosidase in Miller Units (MU) between the regulatory cascade system and the simple systems. (a) Kinetic of  $\beta$ -galactosidase production upon addition of inducer. Salicylate 2 mM was added to the cultures (OD600=0.2), and  $\beta$ -galactosidase activity was monitored at different intervals. Empty symbols: without salicylate. Filled symbols: with salicylate 2 mM; *nahR/Psal::trp'::lacZ* (square), *xylS2/Pm::trp'::lacZ* (triangle), and *nahR/Psal::xylS2/Pm::trp'::lacZ* (circle). (b) Production of  $\beta$ -galactosidase at different salicylate concentrations with *nahR/Psal* (filled circle), *xylS2/Pm* (triangle) and *nahR/Psal::xylS2/Pm* (empty circle).

Figure 5. Capacity for regulation of simple circuits and with two cascades using a different hierarchy of upstream and downstream regulators. S: salicylate 2mM. B: benzoate 2 mM. 3, 5 dClS: 3, 5 -dichlorosalicylate 2 mM. Basal values of  $\beta$ -galactosidase activity from each circuits established in *E. coli* were the followings: *nahR/Psal* (black bars), 65 MU; *xylS2/Pm* (open bars), 192 MU; *nahR/Psal*Ø *xylS2/Pm* (grey bars), 169 MU, *xylS2/Pm*Ø *tnahR/Psal* (hatched bars) cascade circuit was 69 Miller Units. Data are the mean values of three independent experiments. The corresponding standard deviations are shown with the error bars.

Figure 6. Capacity for regulation of single and cascade circuits in *Pseudomonas putida*. Strains bearing in the chromosome different minitransposons (first column) containing the regulatory system described in the legend were assayed for their  $\beta$ -galactosidase accumulation in response to benzoate 2 mM. The fourth column displayed the regulators and sequential order in the corresponding strain. Data are mean values of three independent experiments. Basal values in Miller Units are indicated.

Figure 7. Gene expression analysis of the cascade system in *P. putida* with *nahR4-Psal* as first regulatory system and *xylS/Pm* as the second one. (a)  $\beta$ -galactosidase accumulation without inducer (-), salicylate 2 mM (S), or benzoate 2 mM (B) with the cascade system (left) or the simple system *xylS/Pm::trp':::lacZ* (right). (b) Western blot for the detection of the XylS production in cultures of *P. putida* (*nahR4/Psal::xylS/Pm::trp':::lacZ*) after incubation with different effectors.

## EXAMPLES

**Example 1. Comparison of the overexpression of *lacZ* in a cascade circuit relative to a simple circuit.**

### Example 1.1

#### *Construction of a cascade circuit*

This example illustrates the rational basis, construction and validation of a cascade circuit for gene expression relative to a simple circuit.

To test the efficiency of the amplifier genetic circuits, a plasmid that contains a fusion of *Pm* to *trp':::lacZ* was constructed. A *NotI* fragment with this fusion was cloned into the *NotI* site of pTSPm, resulting in plasmid pTSPm-lacZ. The *Pm* fusion was inserted into the chromosome of *E. coli* CC118 (Herrero 1990) by mating with the donor strain S17-1( $\lambda$ *pir*) that contained the plasmid pTSPm-lacZ in LB-citrate 0.8% at 30EC for 4 hours. The pool of bacteria growing on streptomycin 25 mg/l and rifampicin 50 mg/l was used as recipient in a mating with donor strain *E. coli* S17-1( $\lambda$ *pir*) (pCNB4-S2). To select transconjugants, the mating product was plated onto LB plates with rifampicin, streptomycin and kanamycin (25 mg/l). Ten colonies sensitive to ampicillin (100 mg/l)

were selected for further analysis of  $\beta$ -galactosidase activity (Miller 1972). Cultures from colonies of the transconjugant were grown overnight in LB medium (yeast extract 5 g/l, tryptone 10 g/l, 5 g/l NaCl) at 37°C by shaking. Cultures were diluted 1:100 in fresh media without antibiotic selection and incubated for 2 hours at 37°C. Salicylate (2 mM) was added to the cellular suspensions, which were then shaken at 30°C for 5 hours. Production of  $\beta$ -galactosidase without inducer ranged between 100 and 400 Miller Units depending on the transconjugant. The *lacZ* expression could be dramatically induced when the cultures were induced with salicylate up to 25,000-50,000 MU, which corresponded to a gene expression capacity of 150 to 400 times depending on the transconjugant. Variation in the expression level and capacity among the transconjugants is likely due to the proximity to the origin of replication *OriC* because there are more average gene copies per cell (Sousa et al. 1997).

To compare the expression of *lacZ* from the cascade system with the simple systems that responded to salicylate, *E. coli* CC118RSL9 and CC118FH26 were used that contained insertions of the miniTn5 constructs with the *nahR/Psal::lacZ* and *xylS2/Pm::lacZ* fusions contained, respectively, in the plasmids pCNB4-*lacZ* and pCNB2-*lacZ* (de Lorenzo et al. 1993). The induction ratio in response to salicylate and the absolute values obtained by the simple systems were 10-20 times less than those obtained with the cascade system from CC1184S2PT32. Accumulation of  $\beta$ -galactosidase reached maximum value at 5 hours and the effective rate of synthesis was equivalent to the simple systems (Figure 4a). At 0.5 mM of salicylate concentration the induction ratio reached 90% maximal activity. From the 10 transconjugant strains with the cascade regulatory system, the ones that produced maximal gene expression capacity (CC1184S2PT32) and maximal production of  $\beta$ -galactosidase (CC1184S2PT97) were selected. By SDS-PAGE and densitometry, production of  $\beta$ -galactosidase was estimated to be 9% of total protein in CC1184S2PT32 and about 12% in CC1184S2PT97. In contrast, less than 0.6% of  $\beta$ -galactosidase production was obtained with the simple system.

Table 1. Auxiliary plasmids for cloning into pTSPm

| Plasmid  | Description  | Source                 |
|----------|--|------------------------|
| p18Not   | Equivalent to pUC18 but with a <i>EcoRI-SalI-HindIII</i> cloning site flanked by <i>NotI</i> sites   | (Herrero et al., 1990) |
| pUC18Not | Equivalent to pUC18 but with its MCS flanked by <i>NotI</i> sites  | (Herrero et al., 1990) |
| pVDL8    | Low copy number plasmid derived from pSC101 replicon. It contains the same MCS that p18Not. Recommended for cloning of toxic proteins that would make impossible to maintain in high copy number plasmids such as pUC18Not | (Fernandez et al.1996) |
| pFH2     | Origin of replication of pBR322 and M13 phage. Permits the generation in vivo of N- or C- terminal truncated proteins and provides good prokaryotic TIR for overexpression.  | (Fernandez et al.1996) |

### Example 1.2

#### *Comparison of a cascade system in various configurations with a simple system*

This example illustrates the comparative use of cascade circuits for *lacZ* overexpression in a chromosome versus a plasmid configuration, to compare with simple circuits, and to determine relative stability.

Since the amplification of the expression could increase if the simple regulator/promoter were in a plasmid (due to the increment of gene dose), the relative convenience of using different configurations of activator/promoter to overproduce recombinant proteins was evaluated. To do this, we used the same plasmids miniTn5 but using the strain CC118 $\lambda$ *pir* where they can replicate. The strains with plasmid pCNB4-*lacZ* or pCNB2-*lacZ* showed increments of 10 to 30 fold with regards to the expression in monocopy, indicating elevated gene dose. The comparison of simple (non-cascade) systems in plasmids compared to the cascade system in chromosome indicated that the basal level is 3 to 64 times less than the bacteria with pCNB4-*lacZ* or pCNB2-*lacZ*, respectively. Under induced conditions, the level of  $\beta$ -galactosidase production with the cascade system in chromosome was more than two-fold of the the simple circuit *nahR/Psal* and 50-89% of the level of the *xylS2/Pm* circuit in plasmid. To combine the tight regulation with higher values of product yield, a strain with the regulatory cassette *nahR/Psal::xylS2* that bears a lysogen  $\lambda$ *pir* phage was constructed to allow the replication of pTSPm-*lacZ*. The

plasmid configuration of the fusion of *Pm* to the heterologous gene reached the maximum level of  $\beta$ -galactosidase production and a lower level of basal expression with respect to the strain containing pCNB4-*lacZ*. However, the stability of the cascade system in the chromosomal configuration under conditions of overexpression is 100% after 40-50 generations. In contrast, the plasmid system showed bacterial populations that loses the ability to express *lacZ* in a extent correlating with the level of enzymatic activity in the first five hours of induction. Under induced conditions, the final concentration of viable cells in the chromosomal configuration was about 10 times more than when *lacZ* was overexpressed from plasmids. This observation might be explained by the presence of other genes in the plasmid necessary for replication and maintenance that are also in multicopy and whose expression may cause metabolic burden.

Table 2. Comparison of  $\beta$ -galactosidase activities and stability of the heterologous genes from the cascade and single expression systems in different configurations.

| Strain                        | Plasmid            | <sup>a</sup> $\beta$ -gal activity<br>(Miller Units) |        | <sup>b</sup> % $\beta$ -gal/total proteins |        | <sup>c</sup> <i>lac</i> <sup>+</sup> colonies (%) |        |
|-------------------------------|--------------------|--|--------|--|--------|---|--------|
|                               |                    | - 2OHB   | + 2OHB | - 2OHB                                     | + 2OHB | - 2OHB  | + 2OHB |
| CC118 $\lambda$ <i>pir</i>    | pTSPm- <i>lacZ</i> | 1211   | 1358   | ND <sup>d</sup>                            | ND     | 100   | 100    |
| CC118 $\lambda$ <i>pir</i>    | pCNB2- <i>lacZ</i> | 10917  | 67002  | 3.6  | 13     | 98-81   | 20-1.5 |
| CC118 $\lambda$ <i>pir</i>    | pCNB4- <i>lacZ</i> | 510  | 17952  | ND   | 3.9    | 100-97  | 96-85  |
| CC4S2PMT32                    | -                  | 171  | 30383  | ND   | 8.8    | 100   | 100    |
| CC4S2PMT97                    | -                  | 408  | 39143  | ND   | 11     | 100   | 100    |
| CC1184S2 $\lambda$ <i>pir</i> | pTSPm- <i>lacZ</i> | 1487   | 78257  | ND   | 20     | 100-99  | 5-0    |

Cultures were grown at 37°C in LB with (strains with plasmids) or without ampicillin 150  $\mu$ g/ml. Dilutions 1:100 of the cultures were made in the same media without antibiotic selection and incubated for 2 hours at 37°C.

<sup>a</sup>Series of cell suspensions were incubated then with (+2OHB) or without (-2OHB) salicylate 2 mM final concentration at 30°C.  $\beta$ -galactosidase levels were measured enzymatically and taken for sampling SDS-PAGE after 5 hours.

<sup>b</sup>Measurements of the relative amount of  $\beta$ -galactosidase in cells were made by densitometry of coomassie stained 8% SDS-PAGE.

<sup>c</sup>The stability assay was accomplished by making serial batch cultures without addition of antibiotic and in the presence or absence of salicylate 2 mM at 30°C. After an estimated 40-50 generations, cultures were diluted and plated in LB agar plates with X-gal. Number of blue colonies against white colonies were counted as percentage of strains maintaining the expression system. Three independent experiments were made for each strain and conditions. As expected, deviations of stability values varied considerably among experiments with the plasmid containing strains, likely due to the stochastic appearance of the cured strains during the culture. The extreme values (max. and min.) obtained are shown. Those cases with repetitive results are shown as single values.

<sup>d</sup>Not determined, =0.5%

**Example 2. Use of the cascade system for overexpression of a *lpp'::'ompA'::'phoA* gene within.**

This example illustrates the use of a cascade expression system with an autoreplicative expression vector with the *Pm* promoter to overexpress a recombinant DNA encoding a membrane protein.

Expression of a protein from plasmid can be designed and implemented in a variety of ways and with a variety of genetic backgrounds, as the person of ordinary skill in the art is aware. The plasmids described here demonstrate but one of many possible embodiments. Specifically, the plasmids herein described above need a lysogenic  $\lambda$ *pir* phage to be maintained. To avoid the requirement of the  $\pi$  protein for plasmid maintenance, we constructed a *Pm*-based expression plasmid with a autoreplicative ColE1 replication origin (derivatives) (Fig. 2b).

Plasmid pCCD5 was constructed based on pFH2 (Table I), which includes a versatile MCS that allows easy construction of truncated genes. pCCD5 contains a transcriptional terminator (*rrnBT1*) to reduce readthrough from promoters upstream to *Pm*. Since the whole expression cassette is flanked by *NotI* restriction sites, easy cloning into the same unique site of a variety of mini-Tn5 constructs (de Lorenzo and Timmis 1994), and subsequent introduction of the DNA constructs in strains with the CNB4-S4 minitransposon can be achieved. To test the system, we cloned a hybrid *lpp'-'ompA'-'phoA*

sequence within. This specific fusion encodes an outer membrane protein. These are usually difficult to clone using the traditional expression systems because they damage cells when overexpressed. We used pCCD5 to PCR clone *lpp*'-'*ompA*'-'*phoA* from pTX101 (Francisco et al. 1991) using the oligos 5'-GAGGAATTCAATCTAGAGGGTATTAATA and 5'-CGGGATCCCCGTTGTCCGGACGAGTGCC. The fragment was restricted with *EcoRI* and *BamHI* and inserted into the same sites of pCCD5, resulting in plasmid p5LOA2. A gene encoding alkaline phosphatase *phoA*, was cloned from pPHO7 (Gutierrez and Devedjian, 1989) as a *BamHI* fragment in p5LOA2 (figure 2b). The resultant plasmid, p5LOA2-AP, was introduced in *E. coli* CC1184S2 by transformation. The bacterial cultures with CC1184S2 (p5LOA2-AP) produced more than 20% of the total proteins after addition of salicylate without detection of any product in coomassie-stained SDS-PAGE in uninduced conditions. Maximal protein level was reached after 2-3 hours induction.

**Example 3. Induction of a cascade system using different salicylate derivatives.**

This example illustrates how the effector molecules NahR and XylS2 can synergistically amplify gene expression when integrated properly into a cascade genetic circuit. Exponential cultures of *E. coli* CC1184S2PMT32 (cascade) and simple circuits in *E. coli* strains CC118RSL9 and CC118FH26 were incubated for 5 hours at 30°C with different benzoate derivatives (Table 3).  $\beta$ -galactosidase activity of the cultures incubated with the tested compounds showed that the cascade circuit has more gene expression capacity than the simple circuits (Table 3). It was observed that, in comparison to salicylate, other benzoate derivative molecules, e.g., asantranilate and 5-chloro-salicylate, could also increase  $\beta$ -galactosidase production by at least 10%.

**Table 3. Induction ratio of  $\beta$ -galactosidase activity in the presence of different aromatic compounds**

| Inducer                 | <i>nahR/Psal</i> | <i>xylS2/Pm</i> | <i>nahR/Psal::xylS2/Pm</i> |
|-------------------------|------------------|-----------------|----------------------------|
| Salicylate              | 24               | 13              | 235                        |
| Antranilate             | 22               | 11              | 285                        |
| 2-Acetyl salicylate     | 40               | 10              | 249                        |
| 4-Chloro-salicylate     | 33               | 1               | 191                        |
| 5-Chloro-salicylate     | 32               | 3               | 268                        |
| 3,5-Dichloro-salicylate | 18               | 1               | 147                        |
| 5-Metoxi-salicylate     | 3                | 46              | 183                        |
| Benzoate                | 1                | 44              | 82                         |
| 3-Methyl-benzoate       | 11               | 63              | 240                        |
| 2-Metoxi-benzoate       | 33               | 10              | 160                        |
| 3-Methyl-salicylate     | 17               | 46              | 218                        |
| 4-Methyl-salicylate     | 21               | 7               | 203                        |
| 5-Methyl-salicylate     | 30               | 19              | 257                        |

**Example 4. The efficiency of the amplification cascade depends on the characteristics of the terminal positive regulator.**

This example illustrates that there are specific requirements for the second regulatory system to achieve the amplification of the gene expression capacity by a cascade circuit. Specifically, the activity of the target promoter should be dose-dependent over a broad range of intracellular concentrations of the second (or terminal) regulator.

To see the importance of the regulatory hierarchy in amplification effect, the effect of swapping the upstream and downstream regulators in the XylS2/NahR-based cascade was studied by constructing *E. coli* 2NRSL7. The chromosome of this strain bears the DNA elements *xylS2/Pm::nahR* and *Psal::trp':':lacZ* carried by specialized minitransposons and was thus equivalent to *E. coli* strain CC1184S2PT32 except for the order of regulators in the coupled system. As shown in Figure 5, the capacity of the reverse-coupled system responding to salicylate (24-fold induction) did not increase the capacity over the single *nahR/Psal::lacZ* element. Along the same line, the reversed coupled system of *E. coli* 2NRSL7 was completely insensitive to the XylS2-only effector benzoate. These observations indicate that overexpression of *nahR* does not result in a parallel increase of *Psal* activity, but in a non-productive excess of



the second regulator because the same gene expression capacity from *Psal* could be achieved at a relatively low concentration of NahR. Studies on the mechanism of *Psal* activation indicated that the target site for this activator is occupied regardless of the induction conditions (Huang and Schell, 1991). Thus, the *Psal* promoter appears to depend exclusively on the presence or absence of salicylate and overexpression of *nahR* does not produce higher promoter activity. Taken together, these results indicate that the efficiency of the coupling in a regulatory cascade requires specific properties of the downstream regulator/promoter which include, at least, that the activation of the final target promoter be in a dose-dependent fashion, preferably over a broad range of regulator concentrations. This is true for XylS2 but not for NahR. Thus, the mere sensitivity of the two regulators to the same effector did not grant an augmentation effect unless the appropriate hierarchy was established.

**Example 5. Redesign of the cascade system to amplify gene expression in response to benzoate in *Pseudomonas putida*.**

**Example 5.1**

This example shows that the cascade amplifier system can be designed to respond to signal molecules in microorganisms other than *E. coli*. The design of a specialized *P. putida* strain bearing a benzoate cascade control circuit was performed. The rational of the circuit herein described comprises both a first regulator and a second regulator, each of which responds to benzoate. Since the downstream regulator (XylS2) already responds to this inducer (Fig. 6), the design of a new cascade involved mostly the modification of the upstream regulatory system. To this end, two *nahR* mutants encoding benzoate-responsive variants *nahR3* and *nahR4* were employed using pCNB43 and pCNB44 plasmids, respectively (Cebolla *et al.*, 1997). They were assembled in the coupled systemse *nahR3/Psal:: xylS2* and *nahR4Psal:: xylS2*, along with the reporter segment *Pm:: lacZ*, and then inserted into the chromosome of *P. putida* strain KT2442 to yield *P. putida* 43S2PmL and *P. putida* 44S2PmL. The induction of these strains by benzoate was compared to that of *P. putida* strains bearing either the simple elements NSL7 (*nahR/Psal:: lacZ*), 43L (*nahR3/Psal:: lacZ*), 44L (*nahR4/Psal:: lacZ*) and S2PmL (*xylS2/Pm::*

*lacZ* ), or the cascade with the wild type benzoate-insensitive *nahR* variant 4S2PmL (Fig. 6). The cascade with the benzoate-responsive *nahR* mutants increased from 6-fold to 35-fold the gene expression capacity in response to benzoate compared to the single expression systems with *nahR3*, *nahR4* or *xylS2*. In contrast, the cascade circuit with the wild type *nahR* in 4S2PmL (*nahR/Psal::xylS2*, *Pm::lacZ*) in *Pseudomonas* showed reduced induction capacity by benzoate (~7-fold), due to the absence of response of the upstream regulator to benzoate. These figures matched the predictions raised from the simultaneous induction of each regulator upon acquisition of the ability to respond to benzoate by NahR.

### Example 5.2

This example illustrates experiments designed to eliminate the possibility that the amplification property was only a particular feature of the mutant form of XylS (XylS2) and not of the wild type.

The wild type regulator *xylS*, able to be activated by benzoate but not by salicylate, was used as secondary regulator (Ramos et al. 1986). As a first regulator, we used an effector mutant of the *nahR* regulator, *nahR4*, that is able to recognize benzoate in addition to salicylate (Cebolla et al. 1997). To achieve this, plasmid pNS was constructed by changing the fragment *NcoI* from pCNB1 (de Lorenzo et al. 1993) with the corresponding one of pNS2. A *NotI* fragment of pNS with *xylS* was cloned in the corresponding *NotI* site of plasmid pCNB44 (Cebolla et al. 1997), resulting in plasmid pCNB44-S, that contains *nahR4-Psal::xylS*, and the kanamycin resistance gene within the miniTn5 transposable sequences. The *Pm::trp::lacZ* fusion in pTPm-*lacZ* and that contained in pCNB44-S were inserted in the chromosome of *Pseudomonas putida* KT2442 by conjugation through the donor strain *E. coli* S17-1 $\lambda$ pir, and selected with the appropriate antibiotics. Two transconjugants with the cascade system were tested for their capacity to express *lacZ* with salicylate and benzoate. The expression level was about 4-fold higher with the cascade system than with the simple regulatory system *xylS/Pm* (Fig. 7). These strains also produced about 4-fold more  $\beta$ -galactosidase in the presence of salicylate than with benzoate. This was due to the inability of XylS to respond to salicylate as shown by western blot analysis (Figure 7). Increased

accumulation of XylS was observed when the cultures were induced with salicylate. However, XylS has weak transcriptional activity because there was no effector for XylS. In contrast to XylS accumulation in benzoate, the XylS produced is more active because of the presence of productive effector. Therefore, the presence of a common effector for both regulators is preferred for amplification effect.

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All references cited herein are herein incorporated in their entirety. Those of skill in the art will appreciate that the spirit and scope of the invention are broad beyond the numerous embodiments already described and exemplified herein.

**We claim:**

1. A cascade genetic circuit comprising:  
one or more nucleic acid constructs encoding a plurality of transcriptional regulators, said encoded regulators arranged in a hierarchical order such that expression of an upstream regulator from said plurality stimulates expression of a downstream regulator from said plurality; and  
a final target promoter, said final target promoter responsive in a dose-dependent fashion to a terminal downstream regulator of said plurality of regulators.
2. The cascade genetic circuit of claim 1 further comprising a multiple cloning site downstream of said final target promoter.
3. The cascade genetic circuit of claim 1 wherein at least one of said one or more nucleic acid constructs is present as a chromosomal integration.
4. The cascade genetic circuit of claim 1 wherein at least one of said one or more nucleic acid constructs is present as an autoreplicative plasmid.
5. The cascade genetic circuit of any of claims 1-4 wherein at least one of said plurality of transcriptional regulators is responsive to an inducer.
6. The cascade genetic circuit of claim 5 wherein said inducer is capable of inducing the expression of more than one of said plurality of regulators.
7. The cascade genetic circuit of claim 6 wherein said inducer is a benzoate derivative.
8. The cascade genetic circuit of claim 7 wherein said benzoate derivative is salicylate.

9. A cell comprising the cascade genetic circuit of any of the preceding claims.
10. The cell of claim 9, said cell selected from the group consisting of procaryotic and eukaryotic cells.
11. The cell of claim 10 wherein said cell is a eukaryotic cell selected from the group of eukaryotic cells consisting of mammalian, insect, yeast, and plant.
12. The cell of claim 9 wherein said cell is a bacterial cell.
13. The cell of claim 12 wherein said bacterial cell is a gram-negative bacterial cell.
14. A method of regulating the expression of a nucleic acid sequence, comprising:
  - establishing a cascade genetic circuit according to any of claims 1-8;
  - placing said nucleic acid sequence under control of said final target promoter; and
  - inducing said cascade genetic circuit to stimulate expression of said nucleic acid sequence.
15. The method of claim 14 wherein said nucleic acid sequence encodes a member selected from the group consisting of enzymes, hormones, growth factors, apolipoproteins, therapeutic proteins, diagnostic molecules or proteins, anti-sense molecules, ribozymes, rRNA, tRNA, snRNAs, and portions or derivatives thereof.
16. The method of claim 15 wherein said encoded member is a diagnostic reporter molecule.



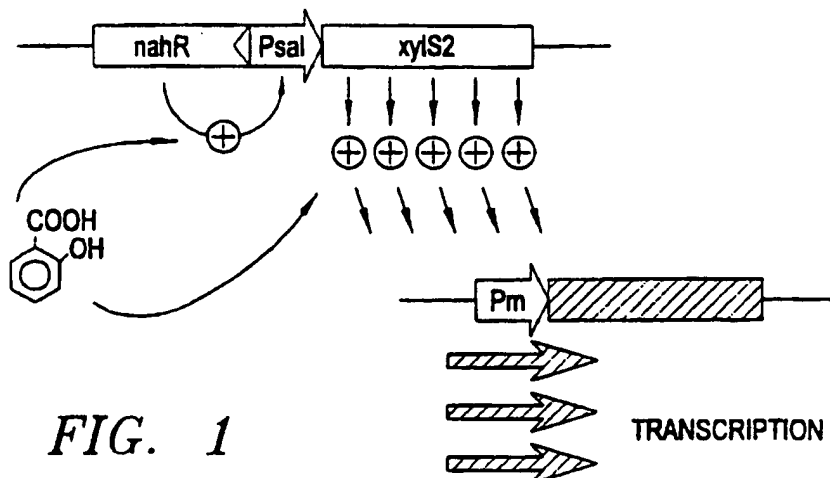
$\frac{1}{4}$ 

FIG. 1

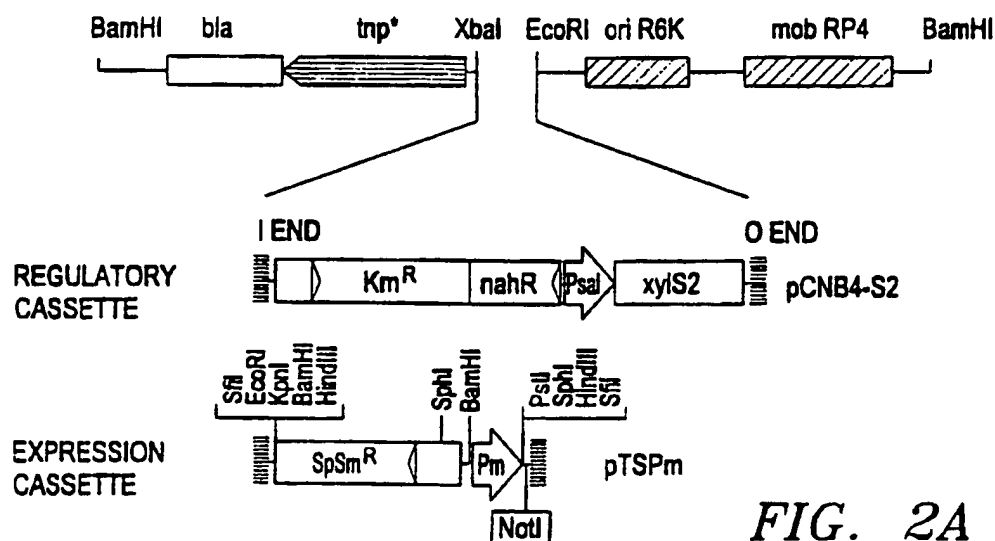
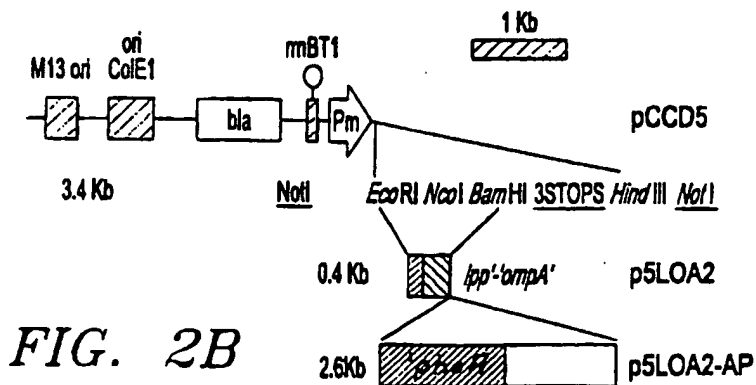


FIG. 2A



*FIG. 2B*

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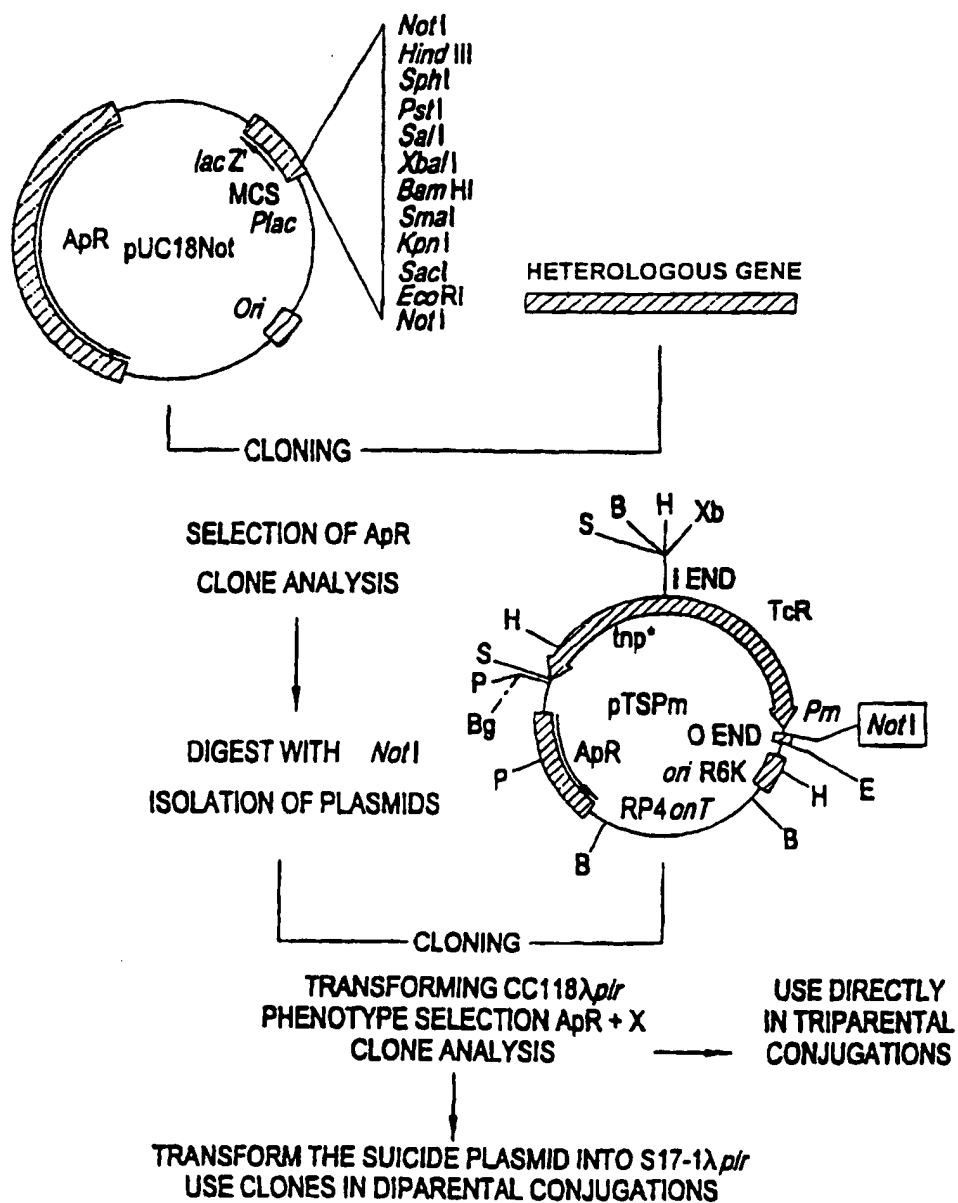


FIG. 3

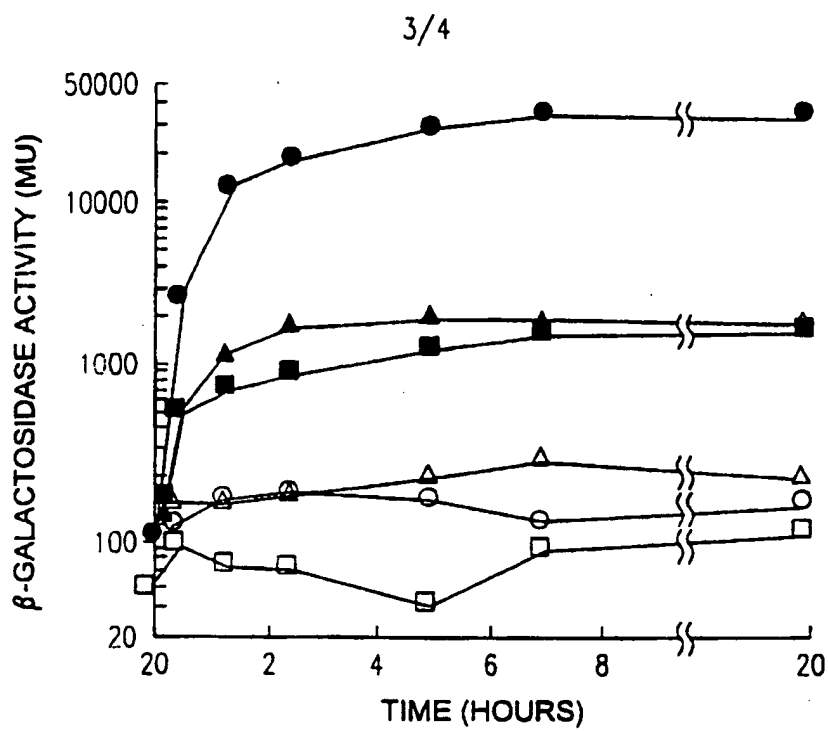


FIG. 4A

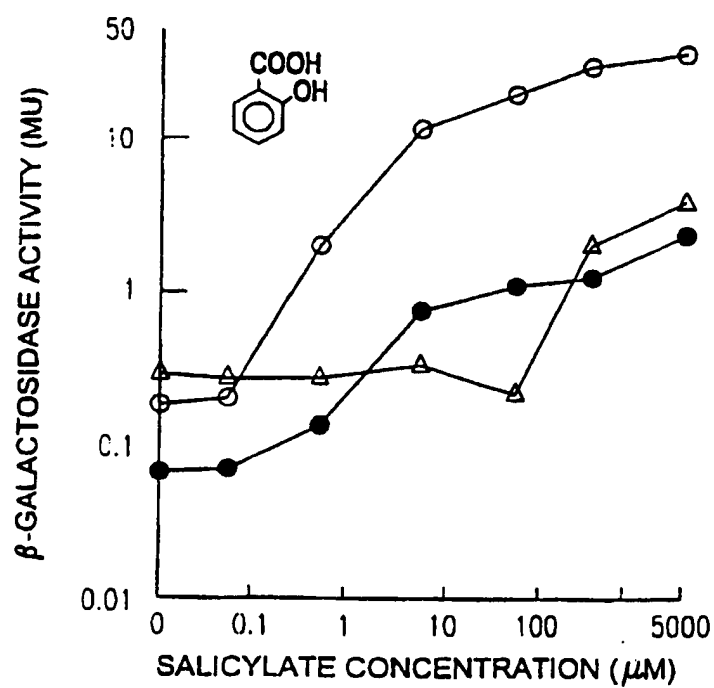


FIG. 4B

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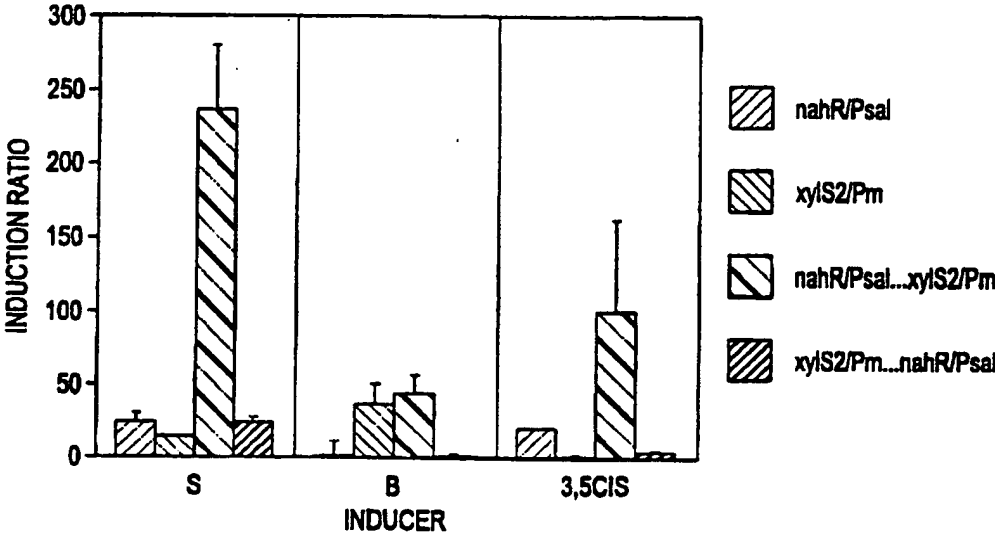


FIG. 5

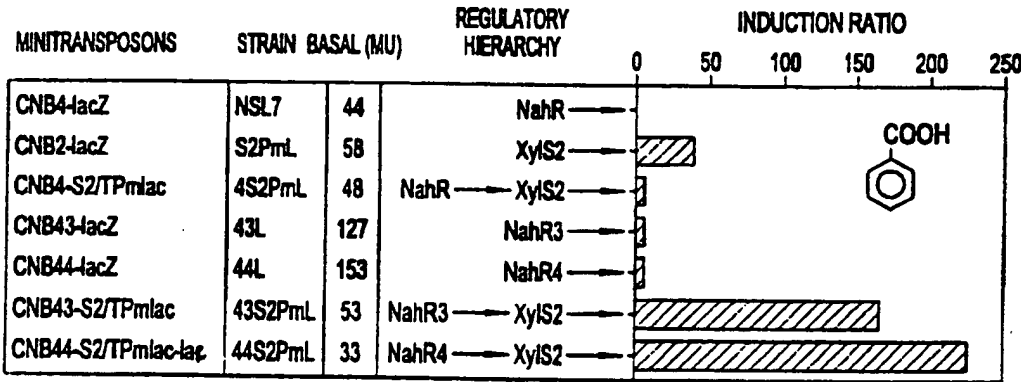


FIG. 6

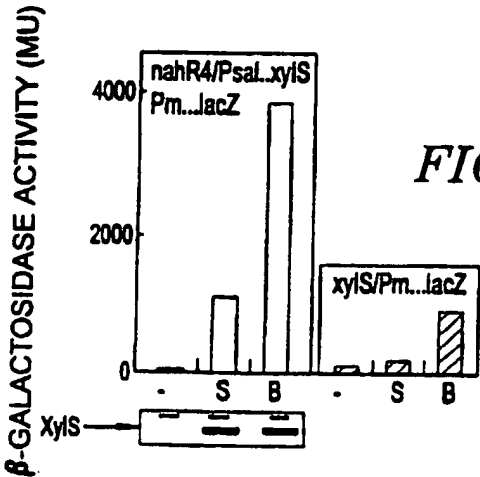


FIG. 7

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 00/00830

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/67 C12N15/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.  |
|------------|--|------------------------|
| X          | SACHIYE INOUE ET AL.: "Overproduction of the xylS gene product and activation of the xylDLEGF opron on the Tol plasmid" JOURNAL OF BACTERIOLOGY, vol. 169, no. 8, August 1997 (1997-08), pages 3587-3592, XP000938324 abstract<br>page 3588, left-hand column, paragraph 3<br>-page 3591, right-hand column, paragraph 1 | 1-15                   |
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

20 September 2000

Date of mailing of the international search report

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.             |
|------------|---|-----------------------------------|
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| X          | <p>RAMOS J L ET AL: "Transcriptional control of the Pseudomonas TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators."<br/> ANNUAL REVIEW OF MICROBIOLOGY, (1997) 51 341-73. REF: 135 ,<br/> XP000938316<br/> page 354, paragraph 2 -page 363, paragraph 2<br/> page 365, last paragraph -page 366, paragraph 5</p>                            | <p>1,4-14</p>                     |
| X          | <p>ANGEL CEBOLLA ET AL.: "Genetic regulation of Nitrogen fixation in Rhizobium meliloti"<br/> MICROBIOLOGIA,<br/> vol. 10, 1994, pages 371-384, XP000938398<br/> page 376, paragraph 5 - paragraph 6;<br/> figure 2</p>   | <p>1,5,6,<br/> 9-11,14</p>        |
| A          | <p>ANTONIO SUAREZ ET AL.: "Stable expression of Pertussis toxin in Bordetella bronchiseptica under the control of a tightly regulated promoter"<br/> APPLIED AND ENVIRONMENTAL MICROBIOLOGY,<br/> vol. 63, no. 1, January 1997 (1997-01),<br/> pages 122-127, XP002147863<br/> cited in the application<br/> page 124, left-hand column, paragraph 1<br/> -page 126, right-hand column, paragraph 1</p> | <p>1-16</p>                       |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

h. International Application No

PCT/IB 00/00830

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)  | Publication<br>date  |
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| WO 9008830 A                              | 09-08-1990          | AU 621195 B<br>AU 4945690 A<br>BR 9007061 A<br>CA 2008700 A<br>EP 0455665 A<br>HU 60777 A<br>HU 215090 B<br>JP 4504500 T<br>RO 112642 A<br>US 5808034 A<br>ZA 9000608 A | 05-03-1992<br>24-08-1990<br>12-11-1991<br>26-07-1990<br>13-11-1991<br>28-10-1992<br>28-09-1998<br>13-08-1992<br>28-11-1997<br>15-09-1998<br>31-10-1990 |